Fluorescence Biochemistry

Enzymatic Incorporation of an Antibody-Activated Blue Fluorophore into DNA**

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Fluorescently labeled DNA polymers are valuable molecular probes that are widely used in fundamental science and medicine. A variety of methods for the generation of fluorescently labeled DNA have been reported over the past 10 years. These include the enzymatic incorporation of fluorophore-conjugated deoxynucleotide triphosphates into nucleic acids by such techniques as random priming, nick translation, and PCR.[1,2] High-throughput genomic analyses such as DNA arrays rely on the availability of fluorescent probes that have exceptional hybridization characteristics, minimal cross-reactivity, and low background fluorescence. Significant progress continues in the discovery of new hybridization assays and in the improvement of those already in use.[3] However, further investigations of new fluorescent nucleic acid probes are required to sustain the current level of success in this field.

In this context we recently reported the generation of antibodies that bind with stilbene to perturb its excited state potential energy surface; upon irradiation with UV light the antibody–stilbene complexes emit a bright blue fluorescence with high quantum yield.[4–6] We have termed such complexes blue-fluorescent antibodies. Aside from their benefit to studies of the dynamics of protein–ligand interactions, these antibodies can be envisioned in a number of practical applications. One such potential application involves modified C-nucleoside–stilbene conjugates designed to probe native and non-natural DNA polymers; we have reported a series of first-generation stilbene-tethered C-nucleosides that have the capacity to combine with monoclonal antibodies (mAbs) that specifically bind *trans*-stilbene. UV irradiation of the resulting complexes produces a powder-blue fluorescence.[5]

Another major potential of blue-fluorescent antibodies lies in the generation of DNA molecules that are essentially

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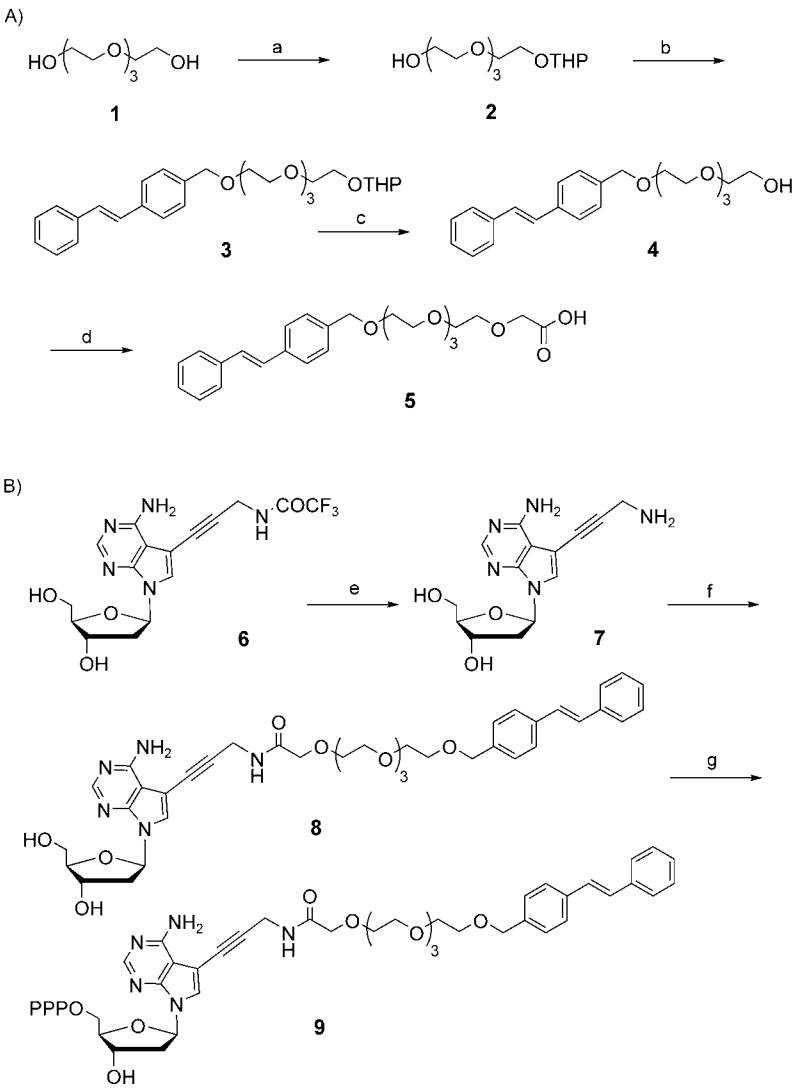
"profluorescent"—they can be activated at will to generate a tunable and long-lived emission. Herein we describe the synthesis of a stilbene-tethered deoxyadenosine triphosphate analogue, its enzymatic incorporation into DNA to yield an antibody-activated blue fluorescent DNA polymer, and hybridization studies of the modified DNA polymer with chromosomal DNA.

To increase the chances of successful enzymatic incorporation into DNA, the stilbene moiety was conjugated to deazaadenosine with a polyethylene glycol (PEG) linker. Deazaadenosine was used for this second-generation conjugate as it is a better mimic of a natural DNA base than is the first-generation substituted benzene C-nucleoside which facilitates its recognition and incorporation by promiscuous DNA polymerases. X-ray crystallographic data from the first-generation stilbene-modified C-nucleoside–antibody EP2-19G2 complex were used for the strategic design of the second-generation linker between the stilbene group and the nucleotide analogue, as it was important to ensure sufficient linker length to allow stilbene protrusion from the DNA helix. These structural data had shown an antibody-binding-pocket depth of 7.5 Å,[6] and a completely buried stilbene molecule. This information in conjunction with the known DNA-major-groove depth of 8.5 Å[5] led to our selection of a PEG linker of four ethylene glycol units. This provides the necessary 16-Å length and is sufficiently hydrophilic to ensure solvent exposure of the stilbene moiety. We therefore anticipated that the PEG linker would permit efficient incorporation by DNA polymerases and facilitate unhindered antibody binding.

Deaza-ATP–PEG–stilbene **9** was synthesized in a convergent fashion as presented in Scheme 1. The stilbene–PEG linker **5** was prepared by coupling chloromethylstilbene with mono(tetrahydropyranyl)-protected tetraethylene glycol **2,** followed by deprotection and addition of bromoacetic acid. With compound **5** in hand, attachment of the deazaadenosine base was the next task. We envisioned the use of **7** to meet our needs as it could be readily prepared from 7-deaza-2′-deoxy-7-[ω-(trifluoroacetamido)propynyl]-adenosine **6**.[7] Conjugation of **5** and **7** through EDC coupling generated **8** as the main product, albeit in moderate yield (Scheme 1B). The stilbene-tethered nucleoside **8** was converted into the final product triphosphate form **9** by a three-step one-pot procedure developed by Kovacs and Otvos.[8] The product **9** in its TEA salt form was purified by DEAE column chromatography and characterized by ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy and FTMS.



Scheme 1. Synthesis of deaza-ATP–PEG–stilbene **9**: A) Synthesis of PEG-modified stilbene: a) dihydropyran, PPTS, CH_2Cl_2, 38%; b) chloromethylstilbene, NaH, THF, 61%; c) *p*TsOH, MeOH, 86%; d) bromoacetic acid, NaH, THF, 67%; B) Conjugation of the PEG–stilbene group to deazaadenosine and phosphorylation: e) NH_4OH, MeOH, 90%; f) **5**, EDC, NHS, CH_2Cl_2/DMF, 36%; g) $POCl_3$, proton sponge, $PO(OMe)_3$; then bis(tri-*n*-butylammonium) pyrophosphate, Bu_3N, DMF; then TEA/H_2CO_3, 52%; DMF = *N*,*N*-dimethylformamide; EDC = 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide; NHS = *N*-hydroxysuccinimide; PPTS = pyridinium 4-toluenesulfonate; TEA = triethylamine; THP = tetrahydropyranyl; Ts = 4-toluenesulfonyl; see Supporting Information for details.

As detailed above, a key prerequisite for the success in the general applicability of compound **9** is for it to be recognized as a viable substrate by thermophilic DNA polymerases. The efficiency of enzymatic incorporation of modified nucleotides into nascent DNA varies widely; it depends on the DNA polymerase used, and the size, charge, and hydrophobicity of the nucleobase modification. It is assumed that the hydrophobic nature of most fluorescent modifications, steric congestion, as well as intra- and intermolecular interactions play an important role in the enzyme-directed incorporation of modified nucleotides into a DNA polymer. Four commercially available thermophilic DNA polymerases were evaluated for their ability to incorporate **9** into a DNA polymer. Based on amino acid sequence similarities to *E. coli* DNA polymerases I, II, III, and IV/V, other DNA polymerases can be classified into families A, B, C, and Y, respectively.[9] From the family of A-type DNA polymerases, Red*Taq* DNA polymerase was chosen; *Vent* DNA polymerase represented the family of B-type DNA polymerases. Critical importance was also placed on the influence of polymerase proofreading (3′→5′ exonuclease activity) on the ability of the enzymes to

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recognize compound **9** as substrate. Therefore, *Pfu* DNA polymerase, known for its efficient proofreading ability was investigated along with an enzyme that lacks proofreading activity, the exonuclease-deficient form of *Vent* DNA polymerase, *Vent* exo⁻.

The gene encoding the anti-cocaine single-chain antibody (scFv) GNC92H2 was chosen as template DNA.[10] It provides an excellent test, as the gene consists of 801 base pairs with 372 adenosine residues, including the flanking primer sequences. In the standard PCR reactions all four natural nucleotides were used at concentrations of 200 μM each, yet the ratio between dATP and **9** was varied (Table 1). The PCR products were purified and subsequently analyzed on an agarose gel (1%) after electrophoretic separation. The results that indicate whether full-length gene products were obtained and the relative yields are summarized in Table 1, and can be summarized as follows: 1) all four commercially-available DNA polymerases are able to incorporate **9** into a DNA polymer; 2) the extent to which these DNA polymerases are able to process compound **9** as substrate depends on the proofreading activity of the enzyme; 3) the proofreading activity appears to interfere minimally with the incorporation of **9**, which suggests that in the case of the *Vent* and *Pfu* DNA polymerases, compound **9** is recognized as a substrate for 3′→5′ exonuclease activity; 4) *Vent* exo⁻ is the most efficient in the incorporation of **9**, and up to 40% of the available ATP in the reaction medium consisted of **9** (although, as measured by UV/Vis and fluorescence spectroscopy (discussed below) the yield of incorporation was lower: roughly 3% of the DNA consisted of **9**). This is consistent with other reports that describe the ability of *Vent* exo⁻ DNA polymerase to incorporate nucleotide analogues.

Table 1: Summary of PCR experiments with different DNA polymerases.

dATP/**9** ratio	98:2	96:4	94:6	92:8	9:1	8:2	7:3	6:4	5:5
[**9**] [μM]	4	8	12	16	20	40	60	80	100
Pfu	√	√	√	√	√	(√)	(√)	–	–
Red*Taq*	√	√	√	√	√	√	(√)	–	–
Vent	√	√	√	√	√	(√)	(√)	–	–
Vent exo⁻	√	√	√	√	√	√	√	√	(√)

√ = PCR product clearly visible; (√) = PCR product visible as faint band; – = no PCR product visible.

It has been shown that *Vent* exo⁻ DNA polymerase has a higher binding affinity for nucleotides and a faster phosphoryl-transfer rate than the *Vent* DNA polymerase.[11] Notably, the overall fidelity of *Vent* exo⁻ DNA polymerase is still twofold higher than that of *Taq* DNA polymerase. To underscore the significance of our findings, we sought to compare the incorporation data of compound **9** with pyrene-8-dATP, a structurally related, commercially available, non-natural fluorescent nucleotide in its role as substrate for the four DNA polymerases tested. Pyrene-8-dATP was also a good candidate for the determination of any potential differences in fluorescence intensity relative to that of **9**; this point is especially pertinent as the fluorescence excitation and emission maxima of pyrene-8-dATP ($\lambda_{excitation} = 340$ nm, $\lambda_{emission} = 376, 395$ nm) are relatively close to those of **9**. Quite unexpectedly, the same four DNA polymerases that incorporated compound **9** successfully, did not produce any observable incorporation of pyrene-8-dATP into nascent DNA along the same DNA template under a wide variety of conditions (Supporting Information).

To further elucidate the properties of the stilbene-modified nucleotide **9** and its corresponding DNA polymer, gel shift mobility assays and sandwich enzyme-linked immunosorbent assays (ELISA) were used to scrutinize purified full-length PCR products containing stilbene residues (Supporting Information). The fluorescence emission characteristics of stilbene-bearing DNA upon binding to mAb EP2-19G2 were measured by fluorescence spectroscopy (Figure 1). The fluorescence quantum yield of the EP2-19G2–**9** complex is lower ($\Phi_f = 0.21$) than the quantum yield of the complex with the nonconjugated stilbene hapten ($\Phi_f = 0.78$). Notably, however, this quantum yield is comparable to that of other fluorescent nucleotides.[2] Furthermore, this fluorescent nucleotide–antibody complex is less prone to bleaching than many other fluorescent nucleotides.

As can be deduced from Figure 1, out of the four polymerases analyzed the *Vent* exo⁻ DNA polymerase was the most efficient in incorporating **9** into DNA. Under the assumption that fluorescence quenching by the DNA polymer is minimal, these results indicate that up to 10

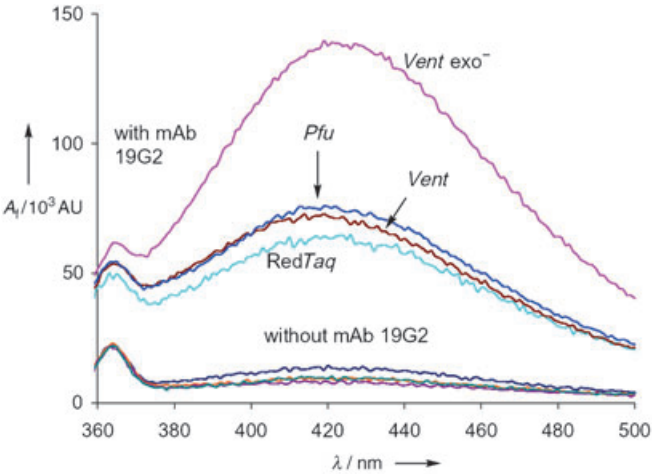

Figure 1. Fluorescence spectra of DNA containing **9** (sDNA). sDNA was obtained by PCR with four different DNA polymerases and a mixture of dATP and **9**, with the template plasmid, pCGMT-92H2. Fluorescence spectra of the purified reaction products (0.1 μM) were measured in the presence (upper four traces) or absence (lower four traces) of excess (10 μM) mAb EP2-19G2. The complex of mAb EP2-19G2 and the stilbene-containing DNA emits blue fluorescence upon UV irradiation ($\lambda_{excitation} = 327$ nm, $\lambda_{emission} = 425$ nm). The signal intensity (with mAb) for the DNA generated by reaction with the *Vent* exo⁻ DNA polymerase roughly corresponds to that of a solution of **9** (1 μM, with mAb) which indicates complex formation of about 10 antibody molecules per stilbene-containing DNA polymer (2.7% of the total number of deoxyadenosine residues present). UV and fluorescence measurements on the sample without added mAb indicated a slightly higher incorporation yield (≈13–14 units of **9** per DNA molecule). Incubation of unmodified DNA with mAb EP2-19G2 showed only background fluorescence.

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stilbene moieties are bound by mAb EP2-19G2. A slightly higher incorporation yield for **9** was observed by spectroscopic analysis of the modified DNA in the absence of antibody (based on the absorption of stilbene at 320 nm); we believe that steric hindrance prevents recognition of some of the neighboring stilbene moieties.

Having established the spectral and enzymatic incorporation properties of compound **9**, the next step was to test the applicability of this technique for hybridization studies. Our initial study was designed to assess the ability of the stilbene-modified DNA to hybridize with isolated chromosomal DNA without loss of its ability to be recognized and activated by an antibody. Isolated genomic DNA from the ovary cancer cell line NIH:OVCAR-3 was used to generate DNA probes specific for the X-chromosomal centromeric alpha satellite DNA, locus DXZ1. NIH:OVCAR-3 cells were grown in culture and harvested at a density of 10^6 mL^{-1}. The cells were then fixed, and hybridization experiments were conducted according to standard literature protocols (Supporting Information). An excess of mAb EP2-19G2 was added, and after an incubation time of 1 h the outcome of the hybridization experiment was analyzed with deconvolution fluorescence microscopy (Figure 2). Figure 2 A provides a differential interference contrast (DIC) image, Figure 2 B shows the fluorescence image of the same view, and Figure 2 C is a merged image of both. The combination of these three images provides strong evidence of hybridization between probes that consist of compound **9** and chromosomal DNA.

We have reported herein the chemical synthesis and biological evaluation of a nucleotide analogue modified with an antibody-activated blue fluorophore. The labeled nucleotide alone is essentially nonfluorescent, and only upon addition of the antibody is fluorescence generated. Thus, the antibody may be envisioned as a secondary affinity reagent that specifically induces a signal. The importance of this is that one can visualize, in real time, the presence or absence of a given strand of DNA by the simple augmentation of a reagent (antibody). This provides an "encoded" response, in which the antibody serves as a means of decoding. This method has the advantage that a fluorescent complex can be generated without the need for a secondary label or antibody that has inherent fluorescence (for example, digoxigenin–fluorescent-antidigoxigenin antibody complexes). All other methods, including continual DNA tagging, present fluorescence properties all of the time.

As a further advantage, the highly specific and strong interaction between the antibody and the stilbene-modified nucleotide should be amenable to DNA affinity purification and enrichment strategies. We anticipate that the preparation of additional non-natural bases tethered to modified stilbenes should allow the generation of a variety of "profluorescent" DNA molecules. In this context we note that very few fluorescent nucleotides fluoresce at 420 nm. This, combined with the ability to change the intensity of the emission, adds variability to the repertoire of techniques available for DNA staining in genomic applications. DNA molecules modified with compound **9** and related bases have envisioned uses in a variety of experimental settings such as investigations of protein–DNA interactions, gene arrays (DNA chips), detection of single nucleotide polymorphisms (SNPs), and in situ fluorescence hybridization (FISH).

Experimental Section

Synthesis. ^{1}H and ^{13}C NMR spectra were measured on a Bruker AMX-400 or Bruker AMX-500 spectrometer as indicated. Chemical shifts (ppm) are reported relative to internal $CDCl_3$ (^{1}H, 7.26 ppm and ^{13}C, 77.0 ppm); CD_3OD (^{1}H, 3.30 ppm and ^{13}C, 49.2 ppm), and [D_6]DMSO (^{1}H, 2.49 ppm and ^{13}C, 39.0 ppm). HRMS data were collected with ESI or MALDI techniques. Glassware and solvents were dried by standard methods. Flash chromatography was performed on silica gel 60 (230–400 mesh), and thin-layer chromatography, on glass plates coated with a layer (0.02 mm) of silica gel 60 F-254. All chemical reagents and solvents were obtained from Aldrich unless otherwise noted, and used without further purification.

8: EDC-HCl was added to a solution of **5** (27.5 mg, 0.062 mmol) and NHS (8.8 mg, 0.074 mmol) in CH_2Cl_2 (1.5 mL) and DMF (0.2 mL), and stirred for 6 h at room temperature under N_2. A solution of **7** (15 mg, 0.05 mmol) in DMF (0.6 mL) was added. The suspension was stirred overnight. After concentration, the residue was purified by PTLC (CH_2Cl_2/MeOH, 10:1) to give **8** (13 mg, 36 %) as a colorless syrup. ^{1}H NMR (400 MHz, CD_3OD): $\delta = 8.05$ (s, 1 H), 7.53–7.46 (m, 5 H), 7.33–7.27 (m, 4 H), 7.23–7.19 (m, 1 H), 7.11 (s, 2 H), 6.43 (dd, $J = 7.9$, 5.8 Hz, 1 H), 4.49–4.46 (m, 3 H), 4.24 (s, 2 H), 4.01 (s, 2 H), 3.99–3.97 (m, 2 H), 3.76 (dd, $J = 12.3$, 3.2 Hz, 1 H), 3.71–3.58 (m, 17 H), 2.62–2.54 (m, 1 H), 2.27 ppm (ddd, $J = 13.2$, 5.9, 2.6 Hz, 1 H); ^{13}C NMR (100 MHz, CD_3OD): $\delta = 173.2$, 159.2, 155.5, 153.2, 150.0,

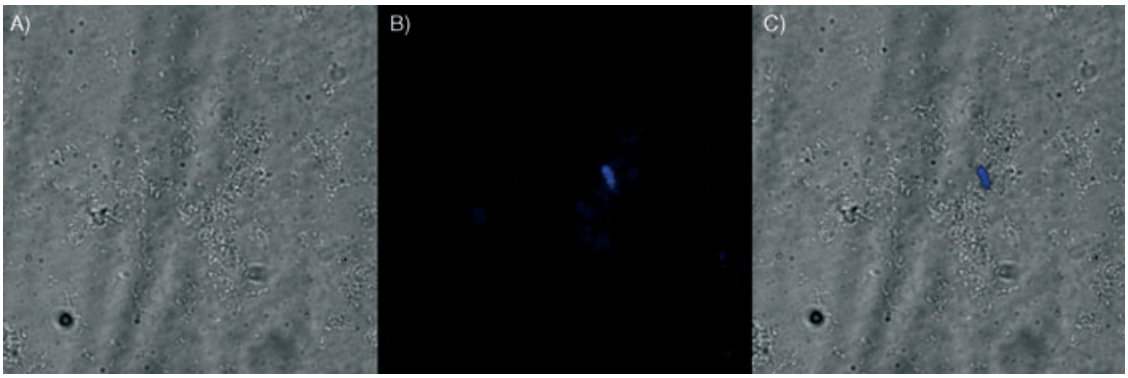

Figure 2. Examples of deconvolution microscopy images showing the hybridization of DXZ1-specific, stilbene-labeled DNA probes to chromosomal DNA. A) Differential interference contrast (DIC) image; B) fluorescence image of the same experiment; C) merged view of images from parts A) and B). Note that a fluorescence image of the sample without added antibody is completely dark.

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139.0, 138.8, 138.2, 130.0, 129.7, 129.3, 129.2, 128.6, 128.0, 127.5, 104.8, 96.9, 89.4, 89.2, 86.7, 76.1, 73.8, 73.0, 72.0, 71.5, 71.4, 71.3, 71.2, 70.6, 63.6, 41.6, 30.2 ppm; FTMS: calcd for $C_{39}H_{47}N_5NaO_9$ $[M+Na]^+$: 752.3266; found: 752.3291.

9: A solution of **8** (13 mg, 0.018 mmol) and proton sponge (5.6 mg, 0.027 mmol) in $PO(OMe)_3$ (0.18 mL) was stirred for 10 min at 0 °C under N_2. $POCl_3$ (3.3 µL, 0.036 mmol) was added, and the mixture was stirred for 2 h at 0 °C. A mixture of Bu_3N (25 µL, 0.11 mmol) and anhydrous bis(tri-*n*-butylammonium) pyrophosphate (40 mg, 0.089 mmol) in DMF (0.18 mL) was added at once. After 1 min, triethylammonium bicarbonate buffer (1.0 M, 4 mL) was added, and the clear solution was stirred at room temperature for 30 min and lyophilized overnight. The crude material was separated by reversed-phase HPLC with a DEAE column (0.1 M TEAB/MeCN) to give **9** (9.0 mg, 52 %) as a white solid. ^{31}P NMR (140 MHz, Tris (50 mM), EDTA (2 mM), pH 7.5 in D_2O): $\delta = -5.6$ (d, $J = 15.8$ Hz), -10.5 (d, $J = 15.8$ Hz), -21.6 ppm (t, $J = 15.8$ Hz); MALDI-FTMS: calcd for $C_{39}H_{50}N_5O_{18}P_3$ $[M+H]^+$: 970.2436; found: 970.2477; TEAB = tetraethylammonium bromide.

Additional experimental details for the synthesis of reported compounds, and ^1H and ^{13}C NMR spectra of compounds **7** and **8** can be found in the Supporting Information.

Fluorescence spectroscopy: Stilbene-containing DNA was generated by using PCR methodology with a dATP/**9** ratio of 7:3 and *Vent* exo$^-$ DNA polymerase. The PCR product was purified, and the product DNA concentration was spectrophotometrically determined to be 0.55 µM. This DNA was used in spectrofluorometric assays to estimate the actual concentration of the stilbene fluorophore. The fluorescence ($\lambda_{excitation} = 327$ nm, $\lambda_{emission} = 425$ nm) of **9** was measured at various concentrations before and after complex formation with mAb EP2–19G2 in PBS (pH 7.4). Compound **9** by itself displayed only negligible fluorescence at the concentrations used (1 µM–50 µM), whereas complex formation with mAb EP2-19G2 resulted in strong blue fluorescence comparable to that measured for the original stilbene hapten complexed with the same antibody.[6] Stilbene-modified DNA (0.1 µM) was incubated with excess (10 µM) mAb EP2-19G2 for 30 min and its fluorescence was measured with an SLM-AMINCO 8100 spectrofluorometer equipped with a 450 W xenon lamp.

Fluorescence microscopy: Fluorescence images were taken with a DeltaVision deconvolution microscope (API, Issaquah WA) equipped with a Photometrics CH350 L liquid-cooled CCD camera attached to an Olympus IX70 inverted microscope. These data were collected with either a 60× (1.4 NA) or a 100× (1.35 NA) oil immersion objective lens, and a DAPI 360/40 nm (excitation), 457/50 nm (emission) filter set. All images were deconvoluted with constrained iterative algorithms (10 iterations) of DeltaVision software (softWoRx, v2.5). The deconvoluted images were subsequently converted into tiff format using softWoRx, v2.5.

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